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# Epitope presentation system based on cucumber mosaic virus coat protein expressed from a potato virus X-based vector

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**Summary.** The *Cucumber mosaic virus* Ixora isolate (CMV) coat protein gene (CP) was placed under the transcriptional control of the duplicated subgenomic CP promoter of a Potato virus X (PVX)-based vector. *In vitro* RNA transcripts were inoculated onto *Nicotiana benthamiana* plants and recombinant CMV capsid proteins were identified on Western blots probed with CMV antibodies 5–7 days post-inoculation. PVX-produced CMV CP subunits were capable of assembling into virus-like particles (VLPs), which were visualized by electron microscopy. We further used the PVX/CMVCP system for transient expression of recombinant CMV CP constructs containing different neutralizing epitopes of Newcastle disease virus (NDV) engineered into the internal βH–βI (motif 5) loop. Both crude plant extracts and purified VLPs were immunoreactive with CMV antibodies as well as with epitope-specific antibodies to NDV, thus confirming the surface display of the engineered NDV epitope. Our study demonstrates the potential of PVX/CMVCP as an expression tool and as a presentation system for promising epitopes.

## Introduction

Cucumber mosaic virus (CMV) is an icosahedral plant virus whose virions consist of 180 copies of the virus-encoded capsid protein (CP). The CMV crystal structure has recently been determined [12]. There are three copies of structurally distinct, chemically identical protein subunits in each icosahedral asymmetric unit. Although the nature of interactions of the viral RNA with the capsids is not clear, the arrangement of a unique hexameric bundle of helices at the N termini of the B and C subunits suggests that the CMV CP might be supported on these structures over the RNA core [12]. It is assumed that successful *in vivo* and *in vitro* assembly of CMV virions depends primarily on protein–RNA interactions and that stability of particles is determined by the presence of viral RNA. CMV

RNAs 1 and 2 are encapsidated separately, whereas one molecule each of RNA 3 and RNA 4 are encapsidated in the same particle [11]. The CMV CP is translated from a subgenomic (sg) RNA 4 located at the 3′ proximal region of the viral RNA 3 molecule.

Development of several CMV-based vectors has been reported elsewhere [8, 10, 15, 16]. In some cases, previous attempts to engineer CMV into a gene delivery vehicle showed instability of the resulting recombinant virus, non-systemic or slow movement of recombinants, and/or symptoms delay [15, 16]. In addition, generation of infectious CMV transcripts, compared to a single-step transcription reaction with the PVX-based vector, requires several additional steps such as separate cloning, linearization, transcription, and co-infection with viral RNAs 1 and 2, significantly complicating the procedure.

A goal of our study was the development of a robust presentation system for effective transient expression of foreign antigenic and therapeutic peptides in plants. In this report, we demonstrate that the coding sequence of the CMV CP ORF placed under the subgenomic promoter of a heterologous plant virus is translated into the CP subunits, which are capable of assembling into CMV virus-like particles (VLPs) in the absence of a full-length RNA 3 molecule and CMV replication machinery associated with RNA 1 and RNA 2. We show that VLPs encapsidate sg RNA encoding the CMV CP and apparently PVX RNA downstream from ORF3 (triple gene block 12 kDa protein) of the hybrid virus. Based on the high-level expression of wild-type (wt) CMV CP from a recombinant PVX template, and assembly of CP subunits into VLPs, we further used the PVX/CMVCP expression system to produce recombinant CMV CP constructs displaying neutralizing epitopes of Newcastle disease virus, an economically important pathogen of poultry. A 17-amino-acid (aa) neutralizing epitope (antigenic determinant II) of NDV's fusion (F) protein (aa 65–81, [14]), an eight-aa neutralizing epitope of the NDV hemagglutinin-neuraminidase (HN) protein (aa 346–353 [3]), and the same epitopes fused together in a tandem F/HN construct were engineered into the βH–βI loop (motif 5) of the CMV CP [6]. Our results indicate that modified CMV CPs carrying these NDV epitopes were recognized by CMV- and NDV-specific antisera, verifying the correct presentation and folding of engineered NDV epitopes that did not disturb their immunogenicity. We propose that the PVX/CMVCP system may be a valuable tool for transient expression of large quantities of useful foreign epitopes including vaccine candidates and antimicrobials as well as a carrier vessel/cage for delivery of other biological materials.

## Materials and methods

### *Plasmid constructions*

The recombinant DNA sequence corresponding to the CMV CP isolate from Ixora (CMV-Ix, [13]) was obtained by PCR-based gene assembly using cloned cDNA of CMV-Ix RNA 3 and the following oligonucleotides: homologous primer LN10, 5′*GATATC*GTTATGGACAAA TCTGAATCAGC3′ (pos. 1255–1277, CMV-Ix RNA3; Italics: *Eco*RV restriction site);

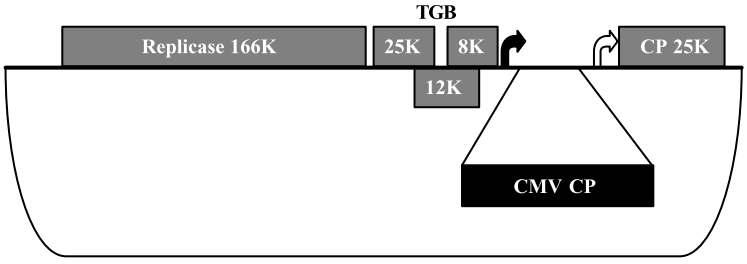



**Fig. 1.** Schematic representation of a PVX vector containing the CMV CP gene. Closed arrow: duplicated PVX CP subgenomic promoter. Open arrow: PVX CP subgenomic promoter. TGB: triple gene block

complementary primer LN2, 5′ACGAGTTCAAACTGGGAGCAC3′ (pos. 1900–1920, CMV-Ix RNA3). The resulting PCR products (672 bp) were cloned into the PCR TOPO II vector (Invitrogen, Carlsbad, CA), digested with *Eco*RV and gel-purified for subcloning into the PVX-based vector pP2C2S [4] (gift of D. Baulcombe, Sainsbury Laboratories, Norwich, England), which had been linearized with *Eco*RV and dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, WI). The recombinant plasmids were used to transform Invitrogen's STBL2 competent cells. The resulting plasmids were designated PVX/CMVCP (Fig. 1). Construction of the recombinant CMV CP, containing F or HN epitopes of NDV, was described elsewhere [16]. The amino acid sequence of the hybrid F/HN epitope of NDV was LLPNMPKDKEACAKAPLDEQDYQIR. The DNA construct, corresponding to the CMV CP with the F/HN epitope incorporated in tandem, was obtained with the following sets of primers: LN10 and AN1, 5′CAATGGTGCCTTTGCGCATGC3′ (complementary to

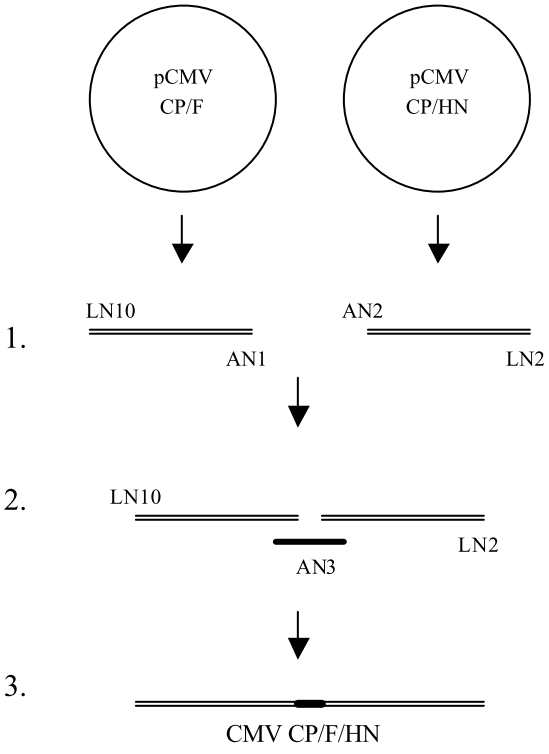



**Fig. 2.** Construction of the CMVCP-F/HN construct by fusion PCR

pos. 31–51 of the NDV F epitope); LN2 and AN2, 5′GATGAACAGGATTACCAAATCC3′ (homologous to pos. 1–22 of the NDV HN epitope). A chimeric, overlapping primer AN3 (5′**GGTAATCCTGTTCATC**CAATGGTGCCTTTGCG3′, bold: complementary to pos. 1–16 of the NDV HN epitope, regular font: complementary to pos. 36–51 of the NDV F epitope) was used in fusion PCR, which also contained products amplified with LN10/AN1 and AN2/LN2 primer pairs (Fig. 2). The resulting PCR products (748 bp) were then cloned into the PVX vector as described above. The resulting plasmid was designated PVX/CMVCP-F/HN. The correctness of all of the recombinant constructs was confirmed by nucleotide sequencing performed on an ABI-PRIZM 373A Genetic Analyzer (DNA Sequencing Facility, Center for Biosystems Research, College Park, MD). Sequence data were analyzed using Lasergene software by DNASTAR (Madison, WI).

### *Transcript preparation and inoculation of plants*

Plasmids were linearized using restriction enzyme *Spe*I, and capped T7-RNA polymerase transcripts were generated from cDNA clones using Ambion's T7 mMessage Machine kit (Ambion, Austin, TX). The transcripts were mechanically inoculated onto fully expanded leaves of *Nicotiana benthamiana*.

### *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

Total nucleic acids were extracted using TRI Reagent (Molecular Reasearch Center Inc., Cincinnati, OH) from non-inoculated symptomatic leaves 5–7 days post-inoculation (dpi). RT and PCR were performed in a single reaction mixture utilizing an RT-PCR Titan One Tube kit as described by the manufacturer (Roche Molecular Biochemicals, Chicago, IL) and the CMV CP-specific primer pair LN2/LN10. The PCR fragments were fractionated on a 0.8% agarose gel.

### *Purification of CMV VLPs and analysis of protein expression by Western blot*

CMV VLPs were purified from 30–100 grams of systemically infected leaf tissue by differential centrifugation using the modified Lot method [9]. Briefly, infected leaf tissues were ground in freshly-made cold 0.5 M Na-citrate, pH 6.5 buffer containing 0.1% thioglycollic acid and 2 ml chloroform per gram of tissue. Extracts were centrifuged for 20 min at 6,000 × g and supernatant was stirred for 15 min with 10% PEG (8000) at 4 °C. The solution was then allowed to sit for 1 h in an ice bath to precipitate the virus, followed by another centrifugation for 20 min at 6,000 × g. The resultant pellet was resuspended in cold 0.05 M Na-citrate buffer (0.5 ml/gram tissue used) containing 2% Triton × 100 and adjusted to pH 7.0. The solution was centrifuged for 5 min at 10,000 × g, the pellets discarded, and the supernatant was centrifuged for 2.5 h at 39,000 rpm (SW-41 rotor). The resulting pellet was resuspended in sterile water overnight at 4 °C and centrifuged 5 min at 10,000 × g. The supernatant contained purified CMV VLPs.

Quantification of protein concentrations in purified samples was performed by a Bradford assay (Sigma, St. Louis, MO). Aliquots were electrophoresed on pre-cast 10–20% Tris-glycine gels (Invitrogen), followed by blotting onto a nitrocellulose membrane (Invitrogen). Alternatively, the gels were stained with Simply Blue$^{TM}$ Safe Stain (Invitrogen). Membranes were probed with polyclonal antibodies specific to CMV CP (American Tissue Culture Collection, Manassas, VA), monoclonal antibodies to the HN epitope of NDV (a gift of R. Iorio, University of Massachusetts Medical School), or antibodies specific to the synthetic F epitope of NDV (Pacific Immunology Corp., Ramona, CA). Reactions were developed with nitroblue tetrazolium chloride (NBT) and Bromo-chloro-indolyl phosphate (BCIP, Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). The concentration of protein in reacting bands

was estimated by comparison of their intensity with positive controls of known concentrations using Alpha Imager software (Alpha Innotech, San Leandro, CA).

### *Immunoelectron microscopy*

The formation of CMV VLPs was visualized by immunoelectron microscopy (IEM). Carbon-coated grids were coated with a 1:10,000-dilution of anti-CMV CP antibodies to capture CMV virions or CMV VLPs, and subsequently treated with anti-CMV CP in a 1:100 dilution to decorate trapped particles. Grids were stained with a 2% water solution of uranyl acetate and examined under a JEOL 100-CXII electron microscope at magnifications of 50,000–100,000. Particle size was measured from recorded images.

### *Protein immunocapture, hybridization assays, and Northern blotting*

Purified preparations of VLPs were loaded on ELISA plates pre-treated with antibodies to CMV (1:1000 dilution) and incubated overnight at 4 °C to specifically immobilize only CMV VLPs. Plates underwent 6–10 × 5 min washing steps with intense agitation to remove any non-specifically bound proteins. Wells were then treated with a 1% Triton X solution in sterile water at 65 °C for 10 min to release RNA from VLPs. Resultant solutions were collected and 1 μl aliquots were applied to a positively charged nylon membrane (Roche) followed by UV crosslinking.

RNA probe synthesis, non-radioactive Digoxigenin (DIG)-labeling, and hybridization conditions were done essentially as advised by manufacturer of non-radioactive DIG-reagents (Roche Molecular Biochemicals). Membranes were hybridized overnight at 68 °C with either CMV CP- or PVX CP-specific DIG-RNA probes. The specificity of CMV CP and PVX CP riboprobes was verified in separate experiments (not shown). A DIG-RNA probe specific for the CMV CP was transcribed from the amplified full-length gene cloned into the TOPO Blunt vector (Invitrogen). PCR primers used for amplification were: LN1, homologous: 5′GTTATGGACAAATCTGAATCAGC3′ (CMV-Ix RNA3 pos. 1255–1276), and LN2, complementary. DIG-RNA probes specific for the replicase, triple gene block (TGB), and CP regions of the PVX genome were transcribed from the cloned PCR products amplified using PVX cDNA as a template with the following sets of primers: RH5, 5′CACCGGTGAGGGC TTGGATAC3′ (pos. 1235–1255, Gene Bank accession number NC_001455) and RH3, 5′CTTTCTTTGCGGCCTGATG3′ (pos. 1645–1663, NC_001455), replicase-derived homologous and complimentary primers, respectively; LN101, 5′ATGTCCGCGCAGGGACA TAGAC3′ (pos. 5147–5168, NC_001455) and LN102, 5′CTCACTAAGGAAGTACTAATG3′ (pos. 5489–5509, NC_001455), TGB-derived homologous and complimentary primers, respectively; LN78, 5′TAGCACAACACAGCCCATAG3′ (pos. 5741–5760, as in pP2C2S); and LN79, 5′GGCAGCATTCATTTCAGCTTC3′ (pos. 6302–6282, as in pP2C2S), CP-derived homologous and complimentary primers, respectively.

Northern blotting was performed using the NorthernMax$^{TM}$-Gly Glyoxal-based system (Ambion).

## Results

### *Infection of N. benthamiana with recombinant PVX/CMVCP virus*

The hybrid PVX/CMVCP vector produced characteristic PVX symptoms – systemic yellowish mosaic and green veins on leaves of *N. benthamiana* 5–7 dpi. Western blot analysis of crude extracts from plants inoculated with the recombinant PVX/CMVCP virus indicated a high level expression of CMV CP (not

shown). The engineered virus remained stable and produced wt CMV CP for at least 7 months after inoculation. PVX/CMVCP did not revert to wt PVX after at least two passages, which was confirmed by continuous expression of the CMV CP.

### *Purification of VLPs*

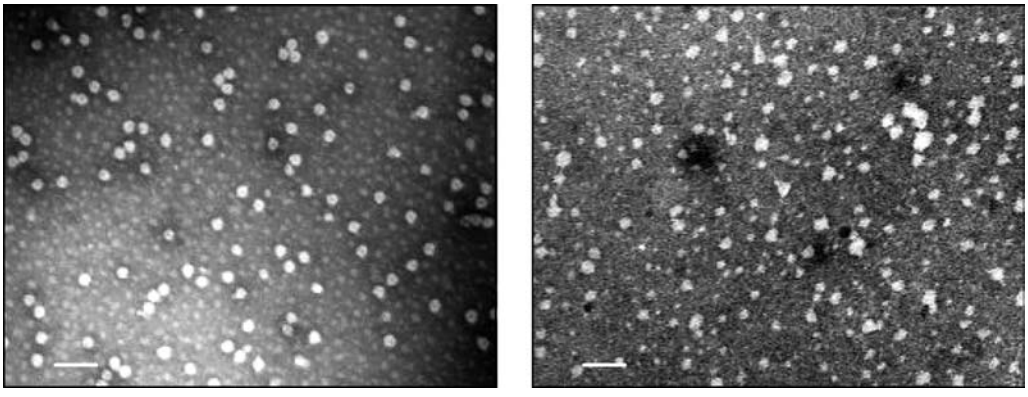
Assuming that PVX-derived CMV CP can assemble into VLPs structurally similar to wt virions, we purified the VLPs using a conventional CMV purification method [9] from systemically infected plants. This procedure resulted in high concentrations of purified VLPs of up to 1–2 mg/ml in the final extract (~0.7 mg/g of tissue).

#### *Immunoelectron microscopy (IEM) of purified samples*

Purified preparations were subsequently tested by IEM using CMV-specific antibodies to capture CMV CP-derived VLPs. An accumulation of CMV-like particles was apparent (Fig. 3), although the particles appear to be of variable sizes compared to a wt CMV preparation, which has uniform-sized particles. The size of the particles, calculated from recorded images, varied between 13–60 nm, with a modal diameter of 33 nm (Fig. 4), which is larger than the 27 nm proposed for the diameter of wt CMV-Ix particles [13]. The majority of VLPs (>50%) belonged to two size groups of approximately 33 and 40 nm.

#### *Analysis of protein expression*

When purified preparations of CMV VLPs were electrophoresed through Novex 10–20% Tris-Glycine gels (Invitrogen) and stained with SimplyBlue$^{TM}$ SafeStain (Invitrogen), a band similar or identical in size to wt CMV CP was present in the CMV VLP sample but not in the sample purified from plants infected with wt



**A** **B**

**Fig. 3.** Immune electron microscopy of CMV VLPs, purified by the Lot method from *N. benthamiana* plants, infected with recombinant PVX/CMVCP virus. **A**, Purified preparation of wt CMV-Ixora. **B**, Purified CMV VLPs derived from PVX/CMV-infected plants. Bars, 100 nm

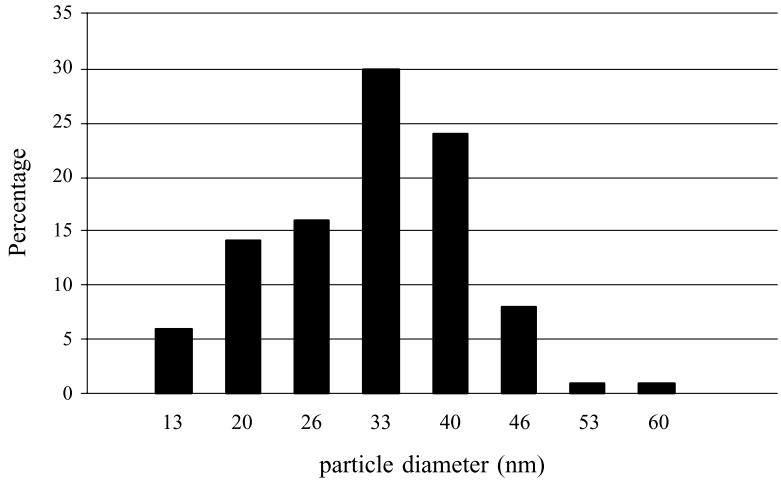



**Fig. 4.** Diameter frequency distribution of CMV VLPs, 100 particles measured

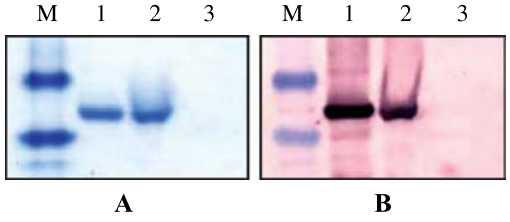



**Fig. 5.** Polyacrylamide gel electrophoresis and Western blot analyses of purified preparations of CMV CP transiently expressed through PVX vector. **A**, Samples stained with SimplyBlue$^{TM}$ SafeStain; **B**, Western blot probed with polyclonal antibodies to CMV. *M*, BioRad pre-stained protein marker; *1*, purified wild type CMV, ~24.2 kDa; *2*, purified preparation of CMV VLPs expressed from PVX vector; *3*, purified preparation from plants infected with wt PVX

PVX (Fig. 5A). Parallel Western blots of the same preparations, probed with polyclonal antibodies specific to CMV CP, showed clear immunoreactivity of the PVX-derived CMV VLPs. The band was immunologically indistinguishable from that of wt CP obtained from CMV-Ix-infected plants (Fig. 5B). There was no reaction with the preparation obtained from plants infected with PVX only. Additionally, Lot-purified VLPs were tested against polyclonal antisera to PVX to analyze homogeneity of the samples. Western blots probed with PVX CP antibodies indicated that some PVX capsids were co-purified along with CMV VLPs (results not shown).

### *Evidence for RNA encapsidation by VLPs*

Northern blotting of RNA extracted from purified samples of CMV VLPs and subsequent hybridization with CMV CP RNA probe suggested the presence of PVX-specific RNA derived from the recombinant virus, in addition to the sg RNA encoding the CMV CP (not shown). These results agreed with our immunoblotting

data, which indicated that a certain amount of co-precipitated PVX capsids was present in the purified preparations of CMV VLPs.

To separate CMV VLPs from co-purified PVX capsids and to accurately determine if they encapsidate subgenomic CMV CP RNA transcribed from PVX vector, we combined a CMV-specific protein immunocapture (IC) and dot hybridization

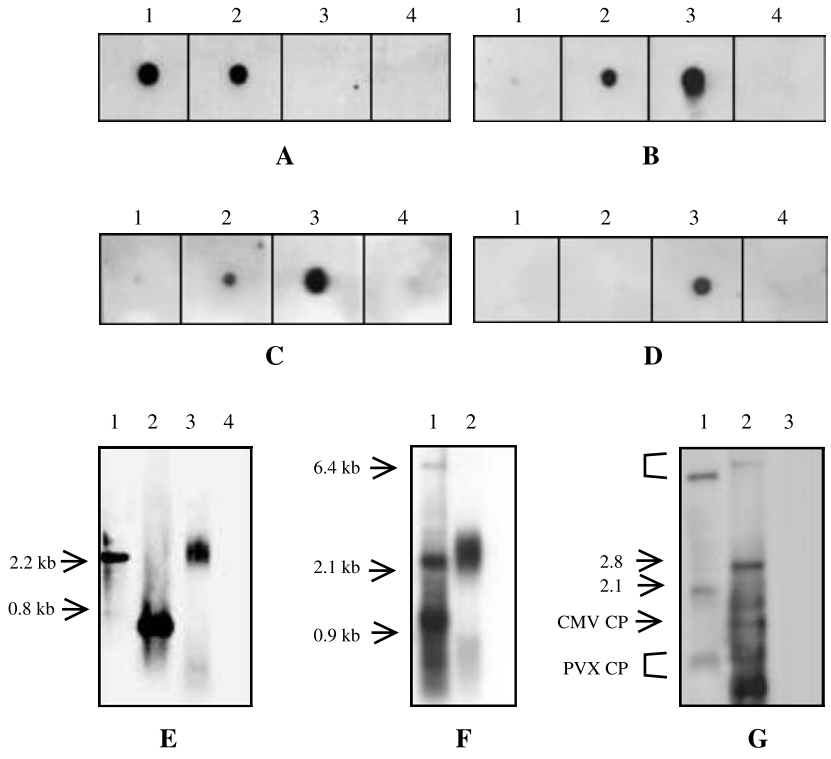



**Fig. 6. A, B, C, D.** Dot-hybridization of RNA samples from immunocaptured (IC)-CMV CP VLPs with Digoxigenin-labeled (DIG) RNA probes. *1*, wt CMV- Ix; *2*, CMV VLPs; *3*, wt PVX; *4*, uninfected control. **A**, Digoxigenin-labeled (DIG) RNA probe specific for CMV CP RNA **B**, DIG RNA probe specific for PVX CP. **C**, DIG RNA probe specific for PVX TGB region. **D**, DIG RNA probe specific for PVX replicase region. **E, F**: Northern blot of RNA samples from IC-purified CMV CP VLPs. **E**, CMV CP-specific DIG RNA probe: *1*, T7 RNA transcript from cloned cDNA of CMV-Ix RNA3; *2*, T7 RNA transcript from cloned cDNA of CMV-Ix CP; *3*, CMV VLPs; *4*, total RNA extracted from uninfected plants. Arrows indicate the sizes of T7 RNA transcripts synthesized from CMV RNA 3 and CP. **F**, PVX CP-specific DIG RNA probe: *1*, total RNA extracted from wt PVX-infected plants; *2*, CMV VLPs. Arrows indicate positions of the PVX genomic and two major subgenomic RNAs. **G**, Northern blot analysis of the total RNA derived from *N. benthamiana* plants, inoculated with wt PVX (*1*) and PVX/CMVCP (*2*). *3* is RNA sample from non-inoculated plant. The membrane was hybridized with DIG RNA probe specific for PVX CP. Positions of the genomic RNAs of wt PVX and PVX/CMVCP are indicated with the upper bracket. The arrows 2.1 and 2.8 indicate positions of the second major sgRNA produced by wt PVX and PVX/CMVCP, respectively. The arrow marked CMV CP indicates the position of extra sg RNAs, produced from duplicated CP SP of PVX/CMVCP. The lower bracket marked CP indicates sgRNAs associated with wt PVX CP and CP of the recombinant PVX/CMVCP virus

with a digoxigenin (DIG)-labeled RNA probe specific to CMV CP ORF. This IC-hybridization technique insured double specificity toward CMV-derived VLPs: first, their separation/capturing based on the antigenic properties and exclusion of any unrelated proteins and second, nucleic acid-supported determination of their RNA content. Dot-hybridization with a CMV CP-specific probe demonstrated that only RNA samples derived from wt CMV and purified VLPs contain CMV CP RNA (Fig. 6A). However, IC-purified VLP preparations also hybridized with the PVX CP-specific probe, indicating that the VLPs might encapsidate PVX CP RNA as well (Fig. 6B). The PVX CP RNA could be derived from sg RNA, which also contains the CMV CP gene and is initiated at the duplicated PVX CP subgenomic promoter (SP) upstream from the inserted CMV CP. To determine if VLPs potentially encapsidate sgRNA originating from the upstream triple gene block (TGB) SP, we hybridized VLP RNA samples with a DIG RNA probe transcribed from the region of PVX cDNA, containing the combined open reading frames 3 and 4 (see Materials and methods). A positive reaction suggested the presence of PVX sg RNA (Fig. 6C). Finally, to determine if full-length PVX RNA non-specifically co-purified with CMV VLPs, we hybridized the VLP RNA with a DIG RNA probe transcribed from the PVX replicase region. The absence of a positive reaction indicated that preparations of IC-CMV VLPs were essentially free of PVX genomic RNA and that its encapsidation by VLPs is unlikely (Fig. 6D). To confirm these findings, we also electrophoresed VLP RNA samples in a 1% agarose gel (NorthernMax$^{TM}$-Gly Glyoxal-based system, Ambion) in order to fractionate RNA, followed by immobilization to nylon membranes and hybridization with the CMV CP or PVX CP DIG RNA probes. To insure a proper size estimation of the VLP RNA, we synthesized RNA transcripts from cloned cDNA of CMV-Ix RNA3 and a CMV-Ix CP RNA and used them as positive controls/markers in a hybridization assay with a CMV CP-derived probe. Total RNA extracted from wt PVX-infected plants was used as a marker in Northern blots with PVX CP riboprobe. An estimated size of the major exposed band, which specifically hybridized with CMV CP probe, was within the range of 2.2–3 kb (Fig. 6E). Hybridization with a PVX CP-specific probe generated a similar-sized band (Fig. 6F). No bands corresponding to PVX genomic RNA were revealed, suggesting that RNA observed in both hybridization assays belongs to IC-purified VLPs rather than to co-purified genomic PVX RNA.

Northern blot analysis of the total RNA derived from *N. benthamiana* plants inoculated with wt PVX and PVX/CMVCP, followed by hybridization with an RNA probe specific for PVX CP, confirmed the presence of sg RNAs associated with recombinant PVX/CMVCP virus (Fig. 6G).

### *Expression of neutralizing epitopes of NDV utilizing the PVX/CMVCP system*

To test the potential of the PVX/CMVCP expression system for the production of foreign antigenic peptides, we designed several constructs of the recombinant CMV CP gene containing different neutralizing epitopes of Newcastle disease

virus (NDV), an economically important pathogen of commercial poultry. The two chimeric CMV CP constructs, containing the eight aa-long neutralizing epitope of the NDV HN gene [3] and the 17 aa-long epitope of the NDV F gene [14] placed in the internal βH–βI loop (motif 5) of the CMV CP, (CMVCP/HN and CMVCP/F, respectively), were essentially as described by Zhao and Hammond [16]. In a third construct, we engineered a tandem F/HN epitope into motif 5 in order to combine two neutralizing epitopes into one expression unit (CMVCP-F/HN, Fig. 7).

*N. benthamiana* plants, infected with the recombinant PVX viruses, showed characteristic PVX symptomatology usually within one week following inoculation with infectious RNA transcripts (not shown). RT-PCR analysis of infected plants using specific primers LN1/LN10, bordering CMV CP gene, showed the presence of mRNA corresponding to each of the chimeric constructs (Fig. 8).

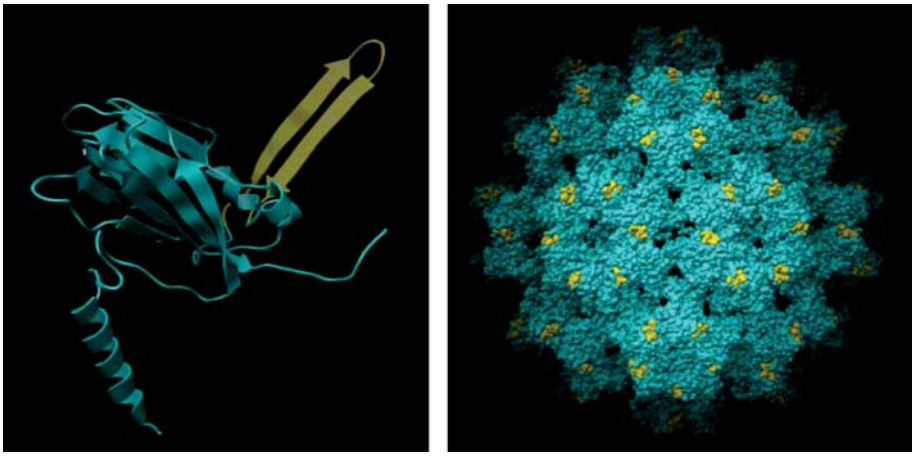



**Fig. 7. A**, A ribbon model of the CMV CP subunit with F/HN epitopes (yellow) of NDV incorporated into motif 5. Predicted structure of the recombinant CMVCP-F/HN subunit generated through the ExPASy proteomics server of the Swiss Institute of Bioinformatics. **B**, A space-filling model of the CMV CP virus-like particle with surface-displayed F/HN epitope (yellow). Both molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081)

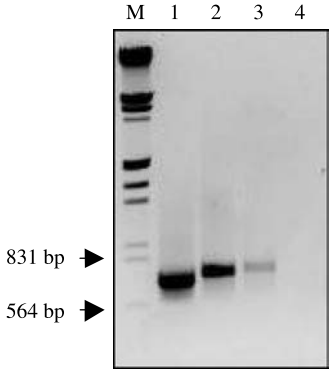



**Fig. 8.** RT-PCR with total RNA samples extracted from plants infected with *1*, PVX/CMVCP/HN; *2*, PVX/CMVCP/F, and *3*, PVX/CMVCP-F/HN, respectively. Lane *4* is an RT-PCR product obtained from non-inoculated plant. *M*, Lambda DNA *Eco*RI/*Hin*dIII DNA Marker (Promega)

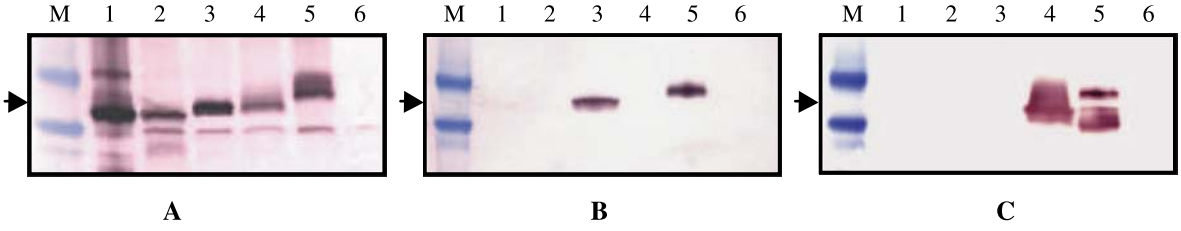



**Fig. 9.** Western blots of purified preparations of CMV CP VLPs transiently expressed from the PVX vector and containing neutralizing epitopes of NDV. *M*, BioRad pre-stained broad range protein marker; *1*, Purified preparation of wt CMV-Ix, ~24.2 kDa; *2*, PVX/CMVCP; *3*, PVX/CMVCP/HN; *4*, PVX/CMVCP/F; *5*, PVX/CMVCP-F/HN; *6*, Uninfected tissue of *N. benthamiana*. **A**: VLPs were probed with polyclonal antibodies to CMV; **B**: VLPs were probed with monoclonal antibodies specific for NDV HN epitope. **C**, VLPs were probed with antisera made against synthetic F epitope of NDV

Both crude plant extracts and Lot-purified VLPs were probed with anti-CMV CP antibodies and also with antibodies specific for the NDV HN or F epitope. Western blots probed with anti-CMV antibodies revealed CMV-CP bands in samples purified from plants inoculated with all constructs: PVX/CMVCP, PVX/CMVCP/HN, PVX/CMVCP/F, and PVX/CMVCP-F/HN (Fig. 9A). VLPs purified from plants inoculated with PVX/CMVCP/HN and PVX/CMVCP-F/HN clearly reacted with monoclonal antibodies to the HN epitope as well as with antibodies produced against the synthetic F epitope, thus confirming proper display and immunogenicity of the engineered epitopes (Fig. 9B and C). A reactive band corresponding to the CMV VLPs displaying NDV HN and F/HN epitopes was also produced from non-purified crude samples (not shown). Electron microscopy examination of the purified preparations revealed the presence of CMV-like particles similar to those extracted from PVX/CMVCP-infected plants (Fig. 3).

Recombinant CMV CPs, containing NDV epitopes, were stably expressed in plants and recognized by specific antibodies for a period of two to three weeks post-inoculation, after which the amount of recombinant protein gradually declined. To assure a good protein concentration needed for the purification of VLPs, a second passage was made three days following the primary inoculation with RNA transcripts, which led to systemic infection 5–7 dpi. No decline in protein concentration was observed if this procedure was applied, and the second-passaged plants were subsequently used for purification of the recombinant antigen.

## Discussion

We evaluated the ability of the CMV CP expressed from a heterologous virus to assemble into stable CMV VLPs, which could serve as a carrier for vaccine epitopes. Encapsidation of subgenomic RNA into the assembling particles was also examined to assess the necessity of RNA-protein interactions for the formation of intact CMV virions. In addition, we demonstrated that the PVX/CMVCP vector system could be successfully used for the expression of foreign antigenic epitopes.

In spite of stability problems encountered with development of the CMV-based vector, viral capsid protein alone represents an attractive candidate as an epitope display system. The internal βH–βI (motif 5) loop within the CMV CP [6] was reported to be a position into which foreign epitopes could be incorporated [1, 16]. Therefore, expression of an engineered CMV CP epitope carrier from a heterologous virus rather than from a CMV-based vector can potentially represent a more stable and productive expression tool. In our study, production of the neutralization epitopes of NDV was easily achievable through the PVX/CMV system and provides a simple and reliable alternative to CMV-based vectors.

We describe here the development of a genetically engineered, self-assembling peptide-carrier vaccine system composed of the CMV CP expressed from the plant-virus expression vector, PVX. Recombinant PVX was infectious, stable, and produced large amounts of heterologous CMV CP, which assembled into CMV-like virions; the VLPs were recognized by CMV-specific antibodies and were immunologically indistinguishable from wt virus. CMV VLPs were easily purified from infected plants in large quantities using a conventional CMV purification method, eliminating the necessity of fusion tags for affinity purification.

Although the purified VLP preparations contained co-purified PVX capsids, their presence, as well as the presence of any host-derived proteins, is not expected to interfere with CMV VLPs' function as an epitope carrier. The PVX capsids do not carry heterologous epitopes and should be tolerated by the immune system as harmless antigens.

Our results indicate a great potential for PVX-derived CMV VLPs as carriers to display antiviral peptides. The NDV epitopes used in this study were immunoreactive with corresponding specific antibodies as well as with CMV CP antisera, demonstrating their correct folding, display and high probability to induce production of neutralizing antibodies *in vivo*.

It has been postulated that successful *in vivo* assembly of CMV virions depends mainly on protein-RNA interactions and that the stability of particles is determined by the presence of viral RNA [1, 7]. It appears that CMV VLPs, produced in plants from the recombinant PVX template, contain not only CMV CP RNA but also package PVX sg RNAs. The presence of variable sizes of CMV VLPs may be due to encapsidation of different sg RNAs of the chimeric virus, similar to the report on tobacco mosaic virus sg RNAs packaged by brome mosaic virus CP [5]. The ability of CMV CP to combine with heterologous RNAs was previously demonstrated [7].

Chimeric VLPs have advantages as antigens. These small particles are highly immunogenic themselves and may serve as an adjuvant to induce a broad immune response to the fused epitope. The presence of a foreign neutralizing epitope in the regular array of a virus structure may stabilize the epitope for a more effective immune response. Besides its application as vaccine antigens, plant VLPs were shown to serve as viral protein cages and biotemplates for nanotechnology, material synthesis, and entrapment [2].

High-level heterologous expression of the CMV CP from the PVX vector and production of compact, assembled VLPs may further contribute both to the

development of vaccine/biomaterials delivery and epitope presentation systems as well as to the study of the biology of CMV, an economically important virus of many crops worldwide.

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